

## **ASSESSMENT OF IMMUNE STATUS, LATENT VIRAL REACTIVATION AND STRESS DURING LONG DURATION BED REST AS AN ANALOG FOR SPACEFLIGHT**

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## **Abstract**

As logistical access for in-flight space research becomes more limited, the use of ground based spaceflight analogs for life science studies will increase. These studies are particularly important as NASA progresses towards the Lunar and eventually Mars missions outlined in the 2005 Vision for Space Exploration. Countermeasures must be developed to mitigate the clinical risks associated with exploration class space missions. In an effort to coordinate studies across multiple disciplines, NASA has selected 90-day bed rest as the analog of choice, and initiated the Flight Analogs Project to implement research studies with or without the evaluation of countermeasures. Although bed rest is not the analog of choice to evaluate spaceflight-associated immune dysfunction, a standard Immune Assessment was developed for subjects participating in the 90-day bed rest studies. The Immune Assessment consists of: leukocyte subset distribution, T cell functional responses, intracellular cytokine production profiles, latent viral reactivation, virus specific T cell levels, virus specific T cell function, stress hormone levels and a behavioral assessment using stress questionnaires. The purpose of the assessment during the initial studies (without countermeasure) is to establish 'control' data against which future studies (with countermeasure) will be evaluated. It is believed that some of the countermeasures planned to be evaluated in future studies, such as exercise, pharmacologic intervention or nutritional supplementation, have the ability to impact immune function. Therefore immunity will likely be monitored during those studies. The data generated during the first three 'control' studies showed that the subjects in general did not display altered peripheral leukocyte subsets, constitutive immune activation, significant latent viral reactivation (EBV, VZV) or altered T cell function. Interestingly, for some subjects the level of constitutively activated T cells (CD8+/CD69+) and virus-specific T cells (CMV and EBV) both decreased during the studies. This likely reflects the isolation of the subjects (from an immunological perspective) and absence of everyday subclinical challenges to the immune system. Cortisol levels (plasma and saliva) did not vary significantly during the studies. This probably reflects a lack of physiological stress during the study and the stress of readaptation to the 1xG environment at R+1. These data demonstrate the absence of significant immune alteration during 90-day bed rest, and establish control data against which future studies (including countermeasures) may be compared.

## Background

Numerous studies have shown that there appears to be a dysregulation of the immune system following spaceflight of varying duration. Specific findings include altered leukocyte distribution and cytokine production postflight (**Crucian 2000, Stowe 1999**) and latent viral reactivation during flight (**Mehta 2000, Stowe 2000, Stowe 2001, Pierson 2005**). There have recently been excellent reviews of what is currently known regarding the status of the immune system during spaceflight (**Borchers 2002, Sonnenfeld 2002**). Although the cause this phenomenon is unknown, a synergy of flight effects such as radiation, physiological stress, or disrupted circadian rhythms are likely causal factors. In addition, microgravity itself may have a direct effect on the function of immune cells, a finding that further complicates flight immune studies.

The goal of NASA and the space life science community is to determine the clinical risks associated with all flight effects on human physiology, so that countermeasures may be developed prior to the initiation of exploration-class space missions. This need has been heightened by the impending lunar/Mars program, soon to be initiated by NASA. Due to the state of the Space Shuttle and ISS programs, it is currently difficult to perform in-flight human physiology research. As mission availability, up-mass volume and crewmember on-orbit science time decreases, the use of ground based spaceflight analogs will increase. The choice of analog largely depends on the physiological system of interest. Closed chamber studies are excellent for psychological research, whereas prolonged bed rest is regarded best for bone and muscle loss. Arctic/Antarctic missions also simulate many aspects of long duration flight, but are a poor analog for bone loss. NASA has recently determined that the bed rest analog represents the most practical model for broad, multi-system spaceflight physiology research. This is in spite of the fact that the bed rest analog is not the best analog for some flight-effects on human physiology, such as immune dysfunction. Although some immune changes have been previously reported using the bed rest analog (altered leukocyte subsets, diminished cellular immunity, altered cytokine profiles), the data vary widely (**Uchakin 1998, Chouker 2001, Gmunder 1992, Schmitt 1996, Schmitt 1996, Schmitt 2000**). In fact, regarding some physiological changes the bed rest model frequently differs from space flight (**Drummer 2000, Norsk 1998, Norsk 2000**).

At the Johnson Space Center, the Flight Analogs Project (FAP) has been initiated and strives to coordinate research spanning multiple human systems, so that data may be shared across multiple disciplines. As such, a panel of immune tests has been included in order to monitor the effect that countermeasures (to be tested in future bed rest studies) may have on immunity. To date, three 'standard' 90-day bed rest studies have been conducted, and the Immune Assessment data are presented in this article. These data are considered 'control' for the future studies where countermeasures such as exercise or medications will be utilized. The assays selected for the FAP Immune Assessment activity determine: (1) general immune status and function; (2) viral specific immunity and viral reactivation; and (3) physiological and psychological stress levels. The specific Immune Assessment panel is presented in [table 1](#).

## Materials and Methods

*Subjects/bed rest.* The 90-day Flight Analogs Project at the Johnson Space Center is outlined in the overview article for this series (**Meck 2006?**). All human study protocols were approved by the Committee for the Protection of Human Subjects of the Johnson Space Center, Houston, Texas, and informed consent was obtained from all subjects. Ten total subjects (5 male and 5 female) participated in three separate 90-day bed rest studies (FAP studies 2, 3 and 4; with 4, 4 and 2 subjects respectively). Unfortunately, whereas studies 2 and 4 lasted the full 90-day duration, study 3 was truncated after ~50 days due to hurricane Rita impacting the Houston/Galveston area. For this reason all data are represented as follows: n=10 for all data points through BR 50; whereas n=6 for all data presented beyond BR 50. For the Immune Assessment activity, whole blood, saliva and 24-hr. urine samples, as well as a psychological questionnaire were collected from all subjects. The complete sampling matrix is presented in [figure 1](#).

*Immunophenotype analysis.* A moderate four-color flow cytometry antibody matrix was created that assessed all the major leukocyte/lymphocyte subsets, as well as activated and memory/naïve T cell subsets. Cell surface markers were stained first combining 100  $\mu$ l of EDTA whole blood and 10  $\mu$ g of each appropriate labeled monoclonal antibodies. Staining was performed by incubated at room temperature for 20 minutes. Red blood cells were lysed using Beckman-Coulter optilyse as described by the manufacturer. Stained leukocytes were then fixed in 1.0% paraformaldehyde in PBS for 10 minutes and analyzed on a Beckman-Coulter Epics XL flow cytometer. The cytometry panel is outlined in [table 2](#). The white blood cell count (WBC) and differential were performed at the indicated timepoints by the Johnson Space Center clinical laboratory using a Beckman-Coulter Hematology Analyzer.

*T cell function.* Immune suppression may consist of a reduced capacity for lymphocytes to respond to stimulus, even though the relative distribution of immune cells is unchanged. For this study the functional response of T cells was measured by activating whole blood cultures in the presence of T cell mitogenic stimuli, followed by measurement of surface activation marker expression on T cell subsets. Culture in the presence of anti-CD3 and anti-CD28 antibodies was used for T cell stimulation. These antibodies activate T cells by triggering T cell surface molecules, requiring the full compliment of intracellular signaling to be utilized (as opposed to phorbol ester or ionomycin). T cell progression through a full activation cycle may be monitored by culturing cells for 24 hours and determining the expression of cell surface CD69 (early activation), CD25 (mid-activation, receptor for IL-2 that requires new gene synthesis).

*Intracellular cytokine analysis.* Lymphocyte and monocyte cytokine production will be assessed for specific cell subsets at the single-cell level utilizing intracellular flow cytometry. The unique advantage of intracellular flow cytometry is the ability to assess the production of multiple cytokines simultaneously in positively identified cell sub-populations using multi-color flow cytometry. Whole blood cultures will be set up by adding 100  $\mu$ l heparin whole blood to 1.0 culture media containing 10 ng/ml PMA, 1.0  $\mu$ g/ml ionomycin and 3  $\mu$ M monensin. Cultures were incubated for 4.0 hours at 37 degrees C. Following incubation, the supernatants were removed, the RBCs lysed as noted above, and the remaining WBCs were fixed in 4.0% paraformaldehyde for 10 minutes. To detect intracellular production of IFN $\gamma$  or IL-2 (following surface marker staining), the fixed PBMC's were resuspended in 200  $\mu$ l of permeabilization buffer, consisting of 5.0% non-fat dry milk and 0.5% saponin in PBS to which 0.5  $\mu$ g of labeled mouse antibody to either IFN $\gamma$  or IL-2 (or both) was added. The cells were incubated at room temperature for 25 minutes and then washed in PBS containing saponin. The cells were then resuspended in 1.0% paraformaldehyde for analysis.

*Plasma viral antibody levels.* Anti-viral antibody titers were determined by indirect immunofluorescence as previously described (**Stowe, 2000, Stowe 2001, Pierson 2005**). Commercially prepared substrate slides and control sera (Bion Enterprises, Park Ridge, IL) were used for determining IgG antibody titers to EBV-viral capsid antigen (VCA) and early antigen (-EA), EBV-nuclear antigen (EBNA), VZV and CMV. Two-fold dilutions of plasma or serum from each subject were prepared, and the endpoint titer was determined as the highest dilution of serum still able to demonstrate immunofluorescence-positive cells. All specimens were batch analyzed and read blind-coded.

**Tetramer assay.** HLA-A\*0201-restricted tetramers (EBV BMLF; CMV pp65) were obtained from Beckman Coulter (San Diego, CA). PBMCs were incubated with PE- or APC-labeled MHC-tetramer complexes, along with CD8-PerCP (BD Biosciences), in dPBS with 2% FCS for 30 minutes at room temperature. Cells were then washed and fixed in 1% PFA and analyzed on a FACSCalibur flow cytometer using CellQuest software for data collection and analysis.

**Peptide stimulation assay.** Intracellular cytokine staining assays were performed as described elsewhere (**Crucian 2001, Komanduri 2001**). PBMCs were isolated from heparinized whole blood by density-gradient centrifugation and washed three times in Dulbecco's phosphate-buffered saline (dPBS) prior to use in functional studies. PBMCs were used immediately and stimulated with HLA-A\*0201-restricted epitopic peptides (10 ug/ml/peptide), controls (PBS) or staphylococcus enterotoxin B (SEB) (10 ug/mL). Peptides used in this study were CMV pp65 (residues 495-503; NLVPMVATV) and EBV BMLF (residues 280-288; GLCTLVAML). Costimulatory monoclonal antibodies (mAbs) – CD28 and CD49d mAbs (1 ug/ml each) (BD Immunocytometry Systems) – were added to each tube. The tubes were vortexed and incubated for 6 h at 37°C with 5% CO<sub>2</sub>, with the addition of Brefeldin A (10 ug/ml; Sigma) for the last 5 h. Following stimulation, cells were washed in PBS, incubated for 5 min at 37°C in 0.02% EDTA and washed in dPBS. Cells were then sequentially incubated for 10 minutes in FACSlyse and FACSPerm solutions (both from Becton-Dickinson Biosciences), washed, and stained with anti-IFN- $\gamma$  (PE), anti-TNF- $\alpha$  (FITC), anti-CD4 or anti-CD8 (PerCP), and anti-CD69 (APC) antibodies. Samples then fixed in 1% paraformaldehyde and analyzed by four-color flow cytometry using a FACSCalibur cytometer and CellQuest software (both BD Biosciences). Flow cytometry data was analyzed and presented using Flow Jo Software (Tree Star, San Carlos, CA); 50,000 events were analyzed for each sample.

**Viral DNA by polymerase chain reaction.** PCR was performed on either urine, saliva, or 1.0x10<sup>6</sup> peripheral blood mononuclear cells. 3.0 ml of each urine sample or 2.0 ml of saliva was concentrated to ~200 uL by centrifugation using a Microsep concentrator 100K (Pall Filtron Corp., Northborough, MA). Extraction of genomic/viral DNA from concentrated urine was performed using the QIAamp Viral RNA Kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. EBV and VZV DNA were quantitated by real-time PCR using an ABI 7700 sequence detector (**Kimura 1999, Mehta 2004, Pierson 2005**). CMV DNA was quantitated using the same methodology but with primers that targeted the immediate early gene (**Tanaka 2000**).

**Stress hormone assessments.** The measurement of hormones has been previously described in detail (**Stowe 1999, Stowe 2001**). Plasma cortisol was measured by EIA, and urinary cortisol was measured by radioimmunoassay. Plasma cortisol was assessed at each timed blood collection and saliva cortisol was assessed for each saliva sample collected (**figure 1**).

**Psychological questionnaire.** Psychological evaluation of all subjects was performed using the Perceived Stress Scale (PSS) and the Positive Affect and Negative Affect (PANAS). Pre-bed rest evaluations were performed every other day from BR-10 to the initiation of bed rest. Evaluations during the bed rest period were performed at the following timepoints: day 37, 39, 41, 43, 45, 47, 49, 51, 75, 83, 85, 87 and 89. Post-bed rest evaluations were performed starting at the end of the bed rest period (BR+0) and continued every other day through BR+12.

**Statistical Analysis.** Due to the low 'n' for this study, some measurements (immune status, function, viral specific immunity) are not statistically analyzed. For these analyses, single subject data or mean data are presented. Where statistical analysis was performed, one-way analysis of variance was used to study significant differences across different times during the study. The method of generalized estimating equations with a logit link was used to find significant difference between the phases (before, during and after the bed rest studies). The differences between sampling periods were considered significant if  $P < 0.05$ .

## Results

**General immune assessment.** The peripheral immunophenotype was assessed for all subjects and consisted of: leukocyte differential, lymphocyte subsets, CD4/CD8 ratio and memory/naïve T cell subsets. The mean leukocyte subset data is presented in [figure 2A](#). No significant changes were observed in any of the subsets measured for the duration of the studies. In addition, no consistent changes were observed for the WBC counts or the relative percentage of neutrophils, basophils or eosinophils (data not shown). Levels of early (CD4/CD69, CD8/CD69) and late (CD4/HLA-DR, CD8/HLA-DR) activated T cell subsets were also assessed in the bed rest subjects. No changes were observed in the levels of CD4+/CD69+ T cells, with levels remaining low throughout the study (data not shown). Constitutive levels of CD8+/CD69+ T cells may range from 2-10 percent in healthy normal subjects. These cells likely represent normal immune activity to everyday environmental challenges. Interestingly, during the bed rest studies, those subjects with a detectable baseline value displayed reductions in the level of CD8+/CD69+ T cells ([figure 2B](#)). This likely is associated with the lack of environmental immune challenges due to the isolation of prolonged bed rest. Among those subjects who had detectable levels (>2.0%) of either CD4+ or CD8+/HLA-DR+ late activated T cells (4 or 8 subjects), the levels decreased during the bed rest study in all cases (data not shown). This data also confirms the lack of immunologic challenge to the subjects immune systems during the bed rest studies.

T cell function was assessed both by intracellular IL-2 and IFN $\gamma$  detection in T cell subsets following PMA/ionomycin stimulation for 4 hours, and CD69+/CD25+ expression following anti-CD3/CD28 antibody stimulation for 24 hours in whole blood culture. The percentage of T cells capable of being stimulated to produce IL-2 tended to vary among the subjects with no mission specific changes evident for either the CD4 or CD8 subset (data not shown). There were no significant mission-associated changes in the percentage of CD4+ T cells capable of being stimulated to produce IFN $\gamma$ , although some subjects displayed reductions by BR 83 ([figure 2C](#)). For most of the full 90-day subjects, there were obvious reductions in the percentage of CD8+ T cells capable of being stimulated to produce IFN $\gamma$  by BR 83 that rebounded sharply after becoming re-ambulatory at BR+1 ([figure 2D](#)). This may be a stress response to re-adaptation. T cell functional assessments of CD69/CD25 expression following mitogenic stimulation did not show any consistent mission-related changes (data not shown).

**Viral specific immunity.** Viral immunity and EBV/CMV reactivation were assessed by determining viral antibody titers (EBV VCA, EBV EA, EBV EBNA and CMV), EBV DNA levels and both the number (via tetramer) and function (via intracellular IFN $\gamma$  following peptide stimulation) of CMV and EBV viral specific T cells. Mean levels of plasma EBV VCA antibody tended to decline over the course of the 90 day bed rest, reaching a minimum at BR+1 ([figure 2](#)). Levels then rebounded to a maximal level at BR+14. Levels of the other EBV and CMV antibody levels did not vary significantly during the bed rest period ([figure 2](#)). VZV antibody titers were measured in the plasma by the indirect immuno-fluorescent method from all the eight subjects before, during and after these studies. No changes in VZV antibody titers were found in any of the subjects from the 60 and 90-day bed rest studies (data not shown). Interestingly, the mean levels of both EBV and CMV specific T cells dropped dramatically from a high point at BR-10, to their lowest point at BR 83 ([figure 3](#)). The levels for both subsets then trended to rise at BR+1 through R+14. The functional capability of the viral specific T cells was determined via viral peptide pulsing followed by intracellular IFN $\gamma$  levels. Although the levels were too small to draw conclusion or significance from, mean levels were slightly elevated at the BR 83 point for both subsets ([figure 3](#)). EBV/IFN $\gamma$  T cells were also elevated at BR+14.

**Latent viral reactivation.** Peripheral leukocyte EBV DNA (assumed to be present in B cells) was assessed to determine active reactivation of latent virus. 9 of the 10 of the bed rest subjects who participated in this study did not display EBV reactivation via this assay (defined as >200 copies per ml). The lone exception was a single 90-day subject who displayed obvious viral reactivation on BR+1 that was not present at BR 83 and had resolved by BR+14 ([figure 4A](#)). EBV copies were also measured in the saliva by real time PCR. There was an increase in the salivary EBV copy number during the study when compared with before and after bed rest ([figure 4B](#)). Two subjects showed peaks of EBV copies in the middle of the study. Their mean + SE values for EBV copies for before were 293 +/- 117.9, during were 803 +/- 303.6 and after the studies (both studies) were 134 +/- 39.6 copies/ ml, however these



differences were not found to be statistically significant. Reactivation of latent VZV was measured by determining VZV copies in the DNA extracted from the saliva collected from all the 10 subjects before, during and after the study. Evidence of VZV DNA was detected only in two of the 10 subjects included in the 90-day Study 3 study (**data not shown**). Urine samples collected from these subjects were analyzed for CMV. CMV DNA was detected in only 3 subjects.

**Physiological stress.** Physiological stress was assessed by determining plasma, and salivary cortisol levels. For salivary cortisol, 5 pre-bed rest samples, 12 mid-bed rest samples, and 8 post-bed rest samples were collected from each subject (**figure 1**). Plasma cortisol was assessed for each blood collection throughout the study (6 total per subject, **figure 1**). For analysis, all pre-, mid-, and post-bed rest values were averaged. Plasma cortisol levels tended to vary among the subjects, and no mission specific changes were detected in any of the subjects (**table 3**). Though there were some sporadic increases in the salivary cortisol levels found during the study (data not shown), the mean values and standard error in both studies did not show any significant changes (**table 3**).

**Psychological stress.** A total of 25 behavioral measurements for Perceived Stress Scale (PSS; **Cohen et. al. 1983**) and Positive Affect and Negative Affect Scale (PANAS; **Watson et. al. 1988**) scores were scheduled to be administered for each subject during the 90-day bed rest studies. No significant difference was found in the Positive Assay score (**figure 5a**), the negative scores (**figure 5b**), and Perceived Stress Scores (**figure 5a**) in these subjects during the full 90-day study as compared to the before bed rest values.

## Discussion

Although the cause of spaceflight-associated immune dysfunction is unknown, it is likely that a synergy of factors such as physiological stress, microgravity, radiation, isolation and an altered microbial environment all contribute to the phenomenon. The various ground based spaceflight analogs may recreate some of these spaceflight factors, but no analog recreates all of them. Bed rest is an excellent analog for the microgravity effects on bone and muscle loss; however the validity of bed rest for immune dysfunction is questionable. Previous bed rest studies that have assessed immunity have reported some immune changes, but the data vary widely between studies and are inconsistent (**Uchakin 1998, Chouker 2001, Gmunder 1992, Schmitt 1996, Schmitt 1996, Schmitt 2000**). The Flight Analogs Project (FAP) was initiated to use a logistically feasible and convenient analog (bed rest) to assess the effects of flight on multiple physiological systems in a coordinated fashion. An assessment of subject immune status was included as a standard measure. The purpose of the immune assessment is two-fold. First, to establish 'baseline' immune data using the standard bed rest analog, against data from future bed rest studies that will include countermeasures may be compared. Future bed rest studies will include countermeasures (such as exercise, nutritional supplementation, vibration, and medication), that all have the potential to influence immunity. Second, the bed rest immune assessment is included to generate data using this analog against which data from other ground-based spaceflight analogs may be compared. There are a multitude of ground-based flight analogs that are likely to be more appropriate with regards to spaceflight-associated immune dysfunction. These include undersea-based NASA Extreme Environment Mission Operations (NEEMO missions) and Canadian Arctic simulated planetary exploration at Houghton Crater on Devon Island (Houghton-Mars Project on Devon Island).

Some of the assays included in this study measure immune changes typically associated with illness or pathology (peripheral leukocyte subset changes), whereas other assays measure the subjects capability to respond when challenged (T cell function, cytokine production patterns). Immune function may be dramatically altered in the absence of pathology or illness, as is expected when otherwise healthy normal subjects experience extreme stress. This phenomenon has been observed in both Arctic and undersea ground based spaceflight analogs (**Crucian 2002, Crucian 2003, Crucian 2004**), marathon runners

(Lakier Smith 2003, Suzuki 2000) and medical students taking exams (Uchakin 2001), and may likely be the situation with astronauts during long duration spaceflight. For this reason, assays selected for the Flight Analogs Project determine: the current immune status; immune functional potential; latent viral reactivation (as an adverse outcome that can be measures); and both physiological and psychological stress levels. Utilizing the major advantage of a ground based spaceflight analog; assessments were performed *during* the bed rest studies at BR 45, and BR 83. The typical BR+0 assessment utilized for most post-flight crewmember assessments was moved for FAP to BR+1. This change was made after consideration that there is no 'landing' event such as the high level re-entry G forces that occurs analogous to following a space mission. The BR+1 assessment was designed to observed the readaptation to unit-gravity that occurs following a bed rest study. Pre-study baseline data (BR-10, BR-2) and post-study 'recovery' data (BR+14) were also assessed for all subjects.

Among the ten bed rest subjects, no changes were seen in the peripheral leukocyte distribution during the studies (**figure 2A**). This is fairly expected, as the subjects were not ill during the studies, and the expansion or contraction of the various immune cell subsets is usually associated with disease. In healthy subjects, the level of constitutively activated (expressing CD69) CD8+ T cells may range from 2-10%. This population likely represents normal subclinical immune activity to everyday environmental challenges. Interestingly, in all four bed rest subjects who completed a full 90-day study, there was an obvious decline in the level of CD8+/CD69+ activated T cells (**figure 2B**). This was unexpected, and is probably the result of being relatively isolated from pathogens and everyday challenges to the immune system for the duration of the 90 days. The decline in peripheral levels of both EBV and CMV viral specific T cells evident throughout the study also lends support to the concept that the subjects were 'isolated' from an immunologic perspective (**figure 3**). It should be noted however, that this assay is restricted to HLA-A2 positive individuals for technical reasons, and unfortunately could only be performed on two subjects. In these data, the subjects were actually 'healthier' during the studies, at least from the perspective of an absence of pathology. It should be noted however, that the goal of the analog is to re-create immune dysfunction, not to induce pathology. Although no obvious changes were observed in T cell functional responses to mitogens and intracellular IL-2 production, there was a definite rise in intracellular IFN $\gamma$  production at R+1 for both T cell subsets (**figure 2C and 2D**). This observation is probably a stress response to the readaptation to the '1xG' environment. Subjects typically are extremely sore for several days following a mission as their bodies re-adapt to bearing weight for the first time 90 days. There was essentially no quantifiable EBV reactivation, as measured by peripheral blood cell EBV DNA (**figure 4A**), however the trend towards an increase in EBV copies in the saliva of these subjects during this study (**figure 4B**) was consistent with previously reported bed rest studies (Pierson and Mehta, 2002), in-flight space studies (**Stowe 2000**) and an undersea ground-based spaceflight analog study (Mehta et al., 2005). Reactivation and shedding of VZV saliva is usually not seen in the healthy human subjects (Mehta et al., 2004). The fact that two subject shed VZV in their saliva indicates the reactivation of VZV did occur under the bed rest condition. The clinical significance of these asymptomatic shedding remains to be tested in future space and space analog studies

Changes in the psychological measures (significant decreases in positive affect, a trend toward higher perceived stress scores) observed in some subjects may suggest an impact on the subjects' mood and to a lesser extent on their level of perceived stress. It should be noted that as a whole these results were quite variable and showed no consistent trend in mood or perceived stress over the duration of the studies. Instances of changes in mood captured by the questionnaire were usually coincident with personal stress-related events unrelated to the study bed rest itself. The bed rest situation creates an artificial isolation environment where it was anticipated that many of life's daily frustrations that may impact overall levels of stress would be eliminated. It was observed however, that this was not the case during these studies. The plasma and salivary cortisol data indicated that no significant physiological stress occurred in these bed rest subjects during these studies.

To summarize, a set of coordinated assays has been developed that is designed to assess: (1) immune status and function; (2) latent viral reactivation; and (3) physiological/psychological stress. This battery of testing is appropriate to measure space-flight associated immune dysfunction in astronaut crewmembers. The testing has been applied to the Flight Analogs Project in an attempt to coordinate multiple-system physiological alterations in an logistically appropriate spaceflight analog. Although



interesting alterations were observed, they probably do not reflect the magnitude of legitimate spaceflight associated immune alterations observed in crewmembers. The data collected during these Studies will, however be used as a 'control' against which future studies will be compared. Several of the future bed rest studies are scheduled to include active countermeasures that may influence immunity. This data will also be extremely useful in comparing the bed rest analog to other ground-based spaceflight analogs.

As NASA (under the auspices of the Vision for Space Exploration) prepares for long duration lunar exploration, and even longer exploration-class missions to Mars it is important that all clinical risks related to spaceflight be established, characterized, and have appropriate countermeasures developed. Despite years of research, immunity remains one of the least-characterized clinical concerns related to flight. This is due primarily to a lack of in-flight sampling. The vast majority of flight immune studies have been post-flight only, usually due to programmatic, logistical or budgetary concerns. While the flight immunology community has done admirable work with these specimens, the postflight data are most likely skewed due the interfering effects of high-G landing and readaptation. Although the perceived immune-related clinical risk associated with short-duration or orbital flight is low, the clinical risk associated with exploration class flight is theorized to be extremely high. This phenomenon is anticipated due to a synergy of factors: much higher energy radiation exposure (and an increased mutagenesis rate), diminished NK cell anti-tumor function, persistent reactivation of Epstein-Barr virus, limited treatment options, and NO Earth-return option. The most sensitive cells in the body to radiation are the marrow immune precursor cells. Considering this, until the necessary in-flight immune studies may be performed, the effective utilization of appropriate ground-based analogs (with mid-mission sampling) is a great start.

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## FIGURE LEGENDS

**Figure 1.** Immune assessment sampling matrix for 90-day bed rest studies. For each study, whole blood, saliva and 24-hour urine samples were collected according to this schedule.

**Figure 2.** Selected general immune status data for subjects participating in 90-day bed rest studies. (A) Mean peripheral blood immune subset percentages for all subjects (n+8); (B) Single subject data regarding constitutive levels of peripheral blood activated CD8+ T cells; (C and D) Single subject data representing levels of CD4+ and CD8+ T cells (respectively) capable of being stimulated to produce interferon-gamma following whole-blood activation in the presence of PMA+ionomycin.

**Figure 3.** Mean subject data representing (A) the peripheral blood plasma levels of EBV and CMV antibodies, and (B) peripheral blood levels of CMV and EBV specific T cells (CMV TET and EBV TET respectively) as well as levels of CMV and EBV specific T cells capable of being stimulated to produce interferon gamma (CMV/IFN $\gamma$  and EBV/IFN $\gamma$  respectively).

**Figure 4.** (A) EBV copies per  $1 \times 10^6$  peripheral blood mononuclear cells; and (B) salivary EBV copies per 1.0 ml saliva during 90-day bed rest studies.

**Figure 5.** Mean positive assay (A), negative assay (B) and Perceived Stress (C) scores before, during and after bed rest study the 90-day bed rest study.

**Table 1: Immune Assessment assays for Flight Analogs Project**

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General Immune Assessment

- Comprehensive immunophenotype
- T cell function
- T cell intracellular cytokine profiles

Viral Specific Immunity

- Viral specific T cell number
- Viral specific T cell function
- Plasma viral antibody levels

Viral Reactivation

- Plasma EBV DNA level
- Saliva EBV/VZV DNA level

Physiological Stress

- Plasma, saliva cortisol levels

Psychological Stress

- PSS score
- PANAS score



Table 2: Flow cytometry panel for the Flight Analog Project

| FL1    | FL2    | FL3  | FL4  | CELL TYPES EVALUATED                  |
|--------|--------|------|------|---------------------------------------|
| CD14   | CD19   | CD45 |      | WBC differential                      |
| CD3    | CD16   | CD19 | CD45 | T cells, NK cells                     |
| CD4    | CD8    | CD3  |      | T cell subsets                        |
| CD45RA | CD45RO | CD8  | CD3  | Memory/naïve T cell subsets           |
| HLA-DR | CD69   | CD8  | CD3  | Early-, late-activated T cell subsets |

Table 3. Cortisol levels (pg/ml) during FAP bed rest studies (mean  $\pm$  standard error).

|                 | <b>PRE-</b>    | <b>DURING-</b> | <b>POST-</b>  |
|-----------------|----------------|----------------|---------------|
| Plasma cortisol | 25 $\pm$ 2     | 26 $\pm$ 2     | 25 $\pm$ 2    |
| Saliva cortisol | 0.98 $\pm$ .07 | .70 $\pm$ .05  | .36 $\pm$ .02 |



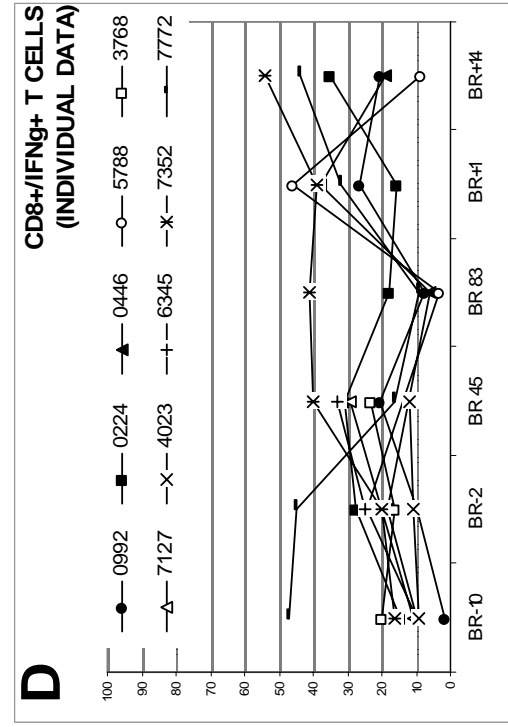
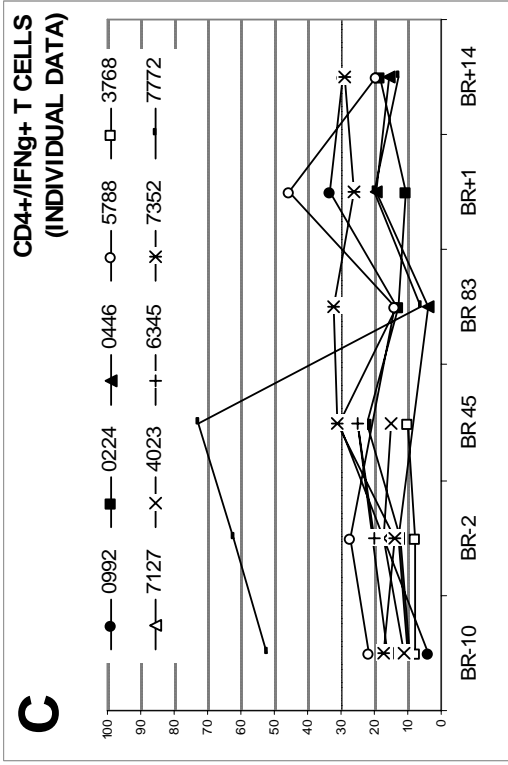
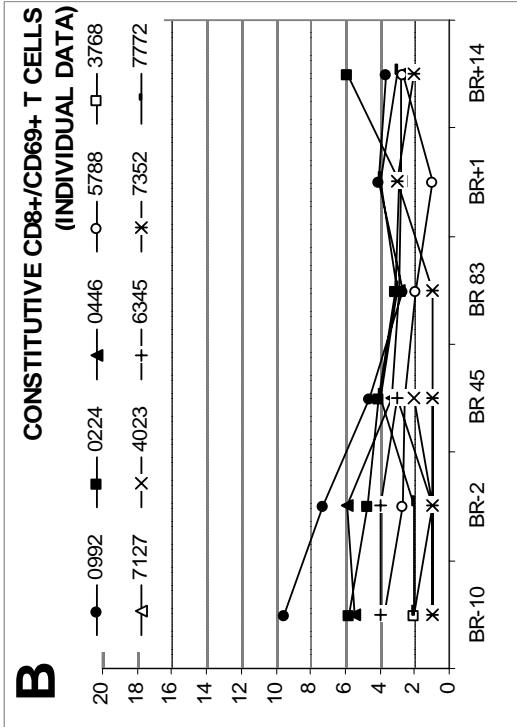
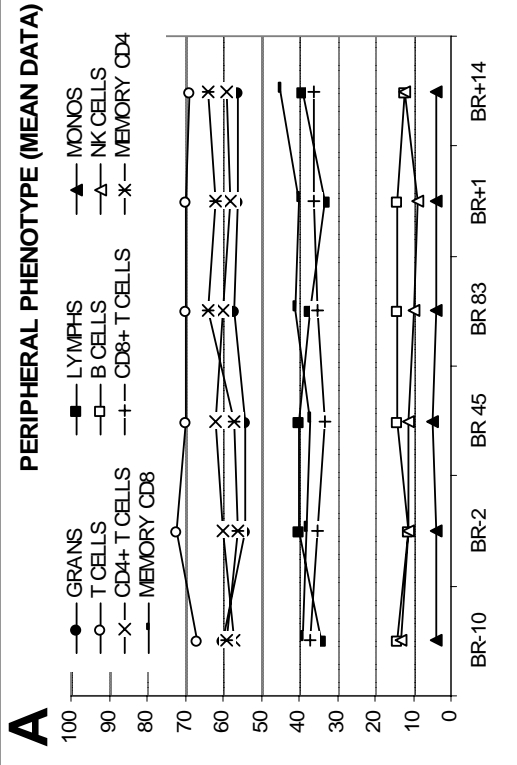


FIGURE 2

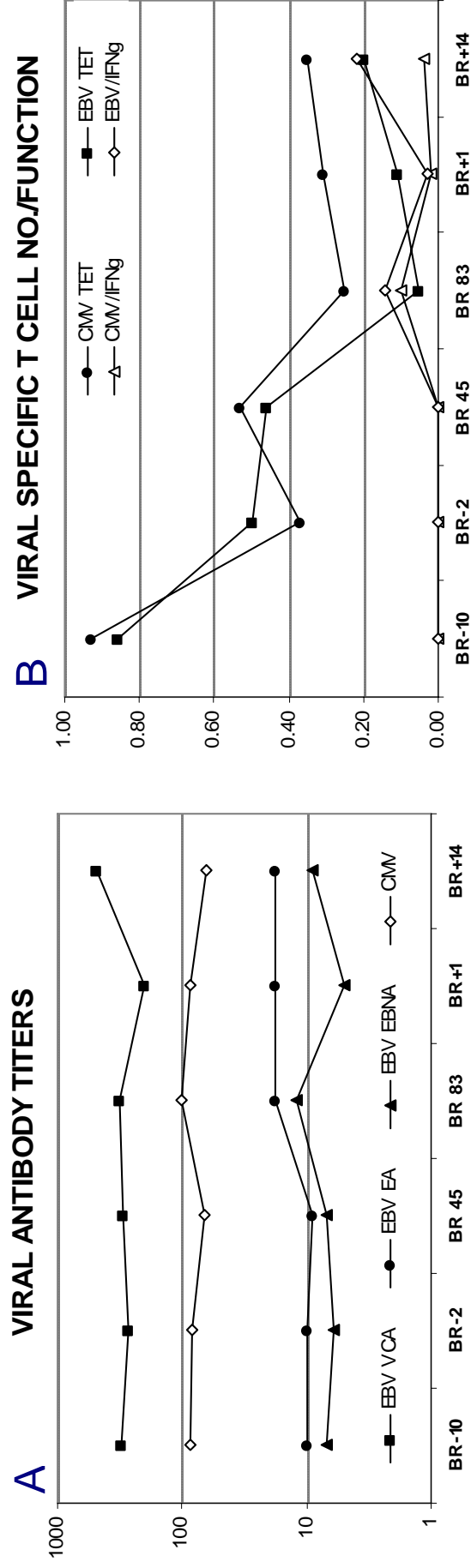
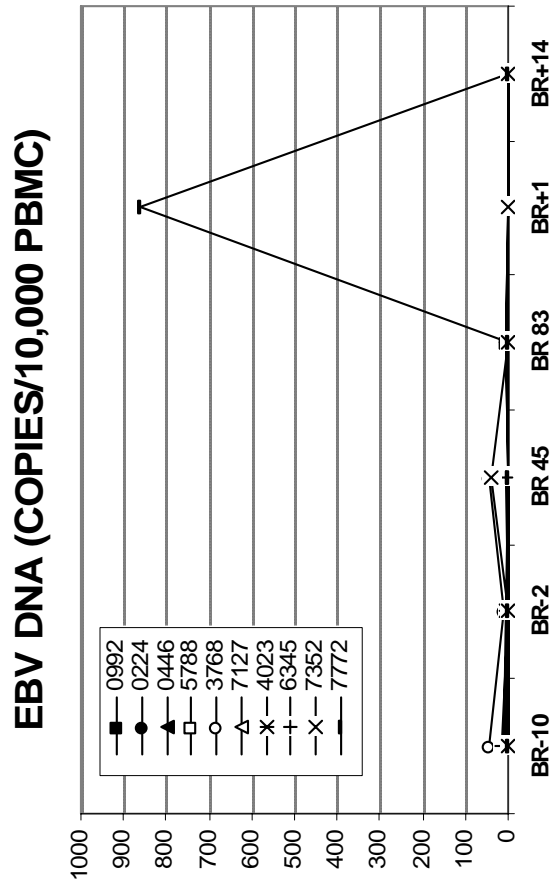
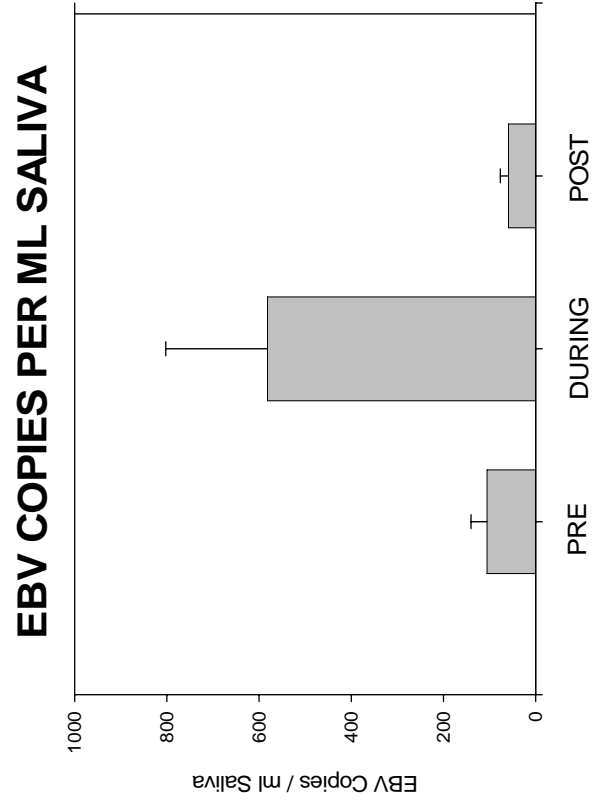
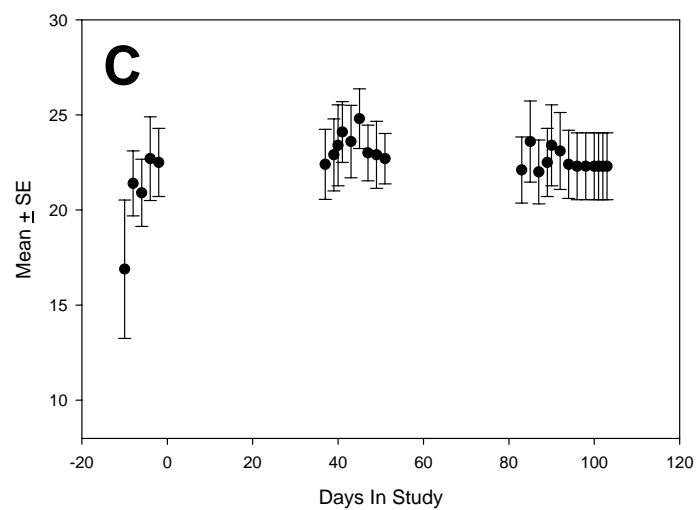
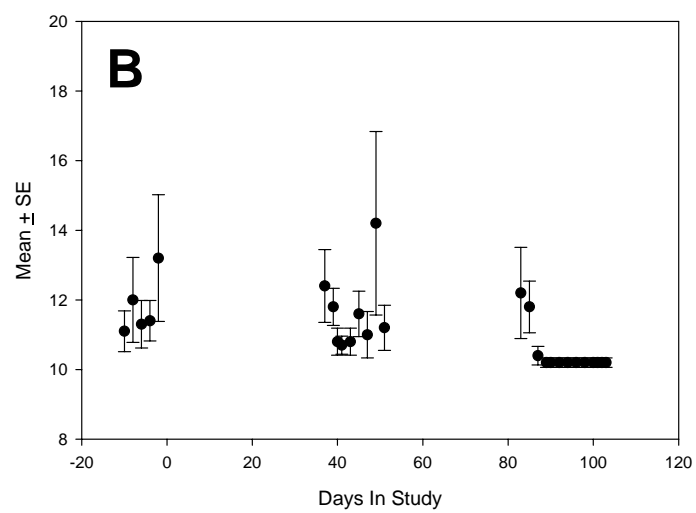
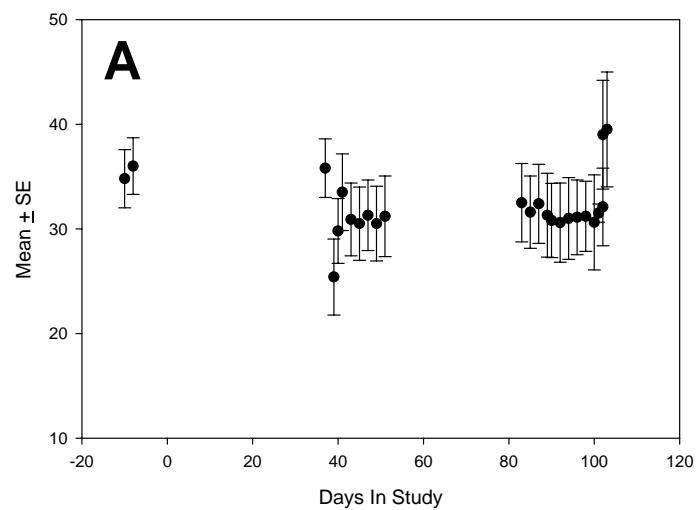


FIGURE 3



**FIGURE 4**





**FIGURE 5**